

Quality of frozen semen of Bali bulls with the addition of Antioxidants Superoxide Dismutase (SOD) and L. Carnitine

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This research aimed at the evaluation of adding antioxidant superoxide dismutase (SOD) and L. Carnitine to the quality of frozen semen Bali bull. A total of 4 Bali bulls aged 9 were used in this research. This research is an experimental research using a complete randomized design (CRD) conducted in two stages, each with 4 treatments and 4 replicates. The first stage was the addition of SOD (control, 0.5 mM, 1 mM, 1.5 mM) and L-Carnitine (control, 0.5 mM, 1 mM, 1.5 mM), and the second stage was the addition of SOD 1.5 mM, L-Carnitine 1 mM, and a combination of SOD 1.5 mM and L-Carnitine 1 mM. The parameters observed for fresh semen include volume, consistency, color, pH, mass movement, motility, and concentration. Meanwhile, parameters observed in frozen semen were motility, abnormality, viability, plasma membrane integrity, acrosome integrity, and DNA integrity. The result showed that the quality of fresh semen of Bali bull was qualified to be processed into frozen semen. Motility, viability, plasma membrane integrity, and acrosome integrity were significantly higher ($P < 0.05$) with the addition of 1.5 mM SOD compared to control, 0.5 mM SOD, and 1 mM SOD. The same thing in the addition of 1 mM SOD was significantly higher ($P < 0.05$) than the control. Still, the addition of 1 mM SOD was not different from the addition of 0.5 mM SOD and the addition of 0.5 mM SOD was also not different from the control. Meanwhile, the abnormality was not different in each treatment but showed a trend of increasing decline until the addition of SOD 1.5 mM. Motility, abnormality, viability, plasma membrane integrity, and acrosome integrity were significantly higher ($P < 0.05$) with the addition of antioxidant L-Carnitine 1 mM compared to control, but not significantly different ($P > 0.05$) compared to the addition of L-Carnitine 0.5 mM, and L-Carnitine 1.5 mM. The same between control, the addition of L-Carnitine 0.5 mM and L-Carnitine 1.5 mM was not significantly different. The addition of SOD 1.5 mM, L-Carnitine 1 mM, and the combination of SOD 1.5 mM and L-Carnitine 1 mM did not affect semen quality. Still, the addition of the combination of SOD 1.5 mM and L-Carnitine 1 mM showed a tendency for higher MPU and TAU and lower DNA integrity. This research concludes that the addition of SOD 1.5 mM and L-Carnitine 1 mM can improve the quality of frozen semen Bali bull.

Keywords: Indigenous livestock, genetic resource, Bali cattle, artificial insemination, frozen semen, thawing, spermatozoa damage.

INTRODUCTION

Bali cattle are developed, utilized, and preserved as an indigenous livestock resource that has certain characteristics and can develop well in various environments in Indonesia. Bali cattle also have quite varied production performance and high reproductive ability. Thus, the genetic resources of Bali cattle are one of the national assets, and they are germplasm that needs to

be maintained and utilized sustainably because they have specific advantages. Bali cattle have also been included in the world's assets, which are recorded in the FAO list as one of the cattle nations in the world (Hikmawaty et al., 2014). The role of Bali cattle is crucial in the development of the livestock subsector, so to increase the productivity of Bali cattle, it is necessary to carry out a mating system by artificial insemination (Hoesni, 2015).

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Increasing livestock productivity can be done through artificial insemination (AI) programs. One of the factors affecting the success of artificial insemination programs is the quality of the semen used. Artificial insemination can be done using fresh or frozen semen. But so far, in practice, it is more dominant to use frozen semen, considering its efficiency in terms of distribution and longer shelf life. Before frozen semen is used for artificial insemination, the quality of frozen semen is checked. This is because, in some cases, processing processes such as semen collection, extender, equilibration, and freezing can affect the quality of frozen semen that will be applied to livestock (Savitri et al., 2014).

Factors that affect the success rate of Artificial Insemination (AI) are selecting the right bulls, the quality and type of bull to be artificially inseminated (AI), semen collection, extender process, semen storage process, semen transportation process, and insemination process. Artificial Insemination (AI) technology is expected to economically provide added value in the development of livestock businesses (Ramon et al., 2021). Based on the Indonesian national standard (SNI 4869-1: 2017), frozen bovine semen, after thawing at 37-38°C for 30 seconds, must show live spermatozoa and progressive (spermatozoa motility) minimum of 40%, a spermatozoa movement score of at least two, and a spermatozoa cell count of at least 25 million per dose (BSN, 2017). The motility value can be used to determine the semen produced so that it can be further processed for cold or frozen storage (Tethool et al., 2022).

Frozen semen refers to semen that has undergone dilution through specific procedures, allowing for extended storage periods while preserving its fertility. However, this method presents a drawback, as the freezing process can compromise semen quality due to exposure to various extreme temperatures, which may lead to a reduction in overall quality (Priyanto, 2015).

The low quality of frozen semen is generally caused by spermatozoa damage caused by improper handling of the freezing process. The most critical part of the semen freezing process is the freezing itself and thawing. During freezing, there is a massive release of water molecules from the cell, resulting in increased intracellular electrolyte concentrations and also the formation of ice crystals. Meanwhile, during thawing, the semen experiences severe stress due to a drastic increase in temperature. Furthermore, an increase in metabolic activity increases the concentration of free radicals, which is where free radicals are very dangerous for spermatozoa survival (Rizal & Herdis, 2010).

One of the significant factors that causes the damage of spermatozoa throughout the cryopreservation process and subsequent thawing is lipid peroxidation. This peroxidative process changes the structural integrity of spermatozoa,

particularly affecting the membrane and acrosome, leading to a decline in motility, accelerated metabolic changes, and the release of intracellular components. The addition of antioxidants to the semen diluent has been shown to mitigate these detrimental effects (Feradis, 2009).

Antioxidants are compounds that can eliminate, clean, and resist the formation of Reactive Oxygen Species (ROS). Antioxidants can reduce, quench, or suppress free radical reactions. Antioxidants can prevent the occurrence of lipid peroxidation reactions in the plasma membrane of spermatozoa during semen processing so that the plasma membrane remains integrity (Wardhana et al., 2012). The more antioxidants added to the semen, the more hydrogen atoms are released, thus better able to prevent lipid peroxidation by converting hydrogen atoms into peroxy radicals. Lipid peroxidation will cause structural damage and disrupt sperm metabolism, leading to sperm death (Hartono, 2012).

Carnitine is an amino acid derivative that enhances energy production and acts as an antioxidant for sperm cells. The fact that L-carnitine is found in high concentrations in the epididymis, the organ where sperm cells mature, increase motility, and utilize fatty acids and phospholipids as energy substrates, suggests that L-carnitine has an important role in sperm cell energy metabolism. Sperm cells require energy from mitochondria for spermatozoa motility (Balogun et al., 2022). L-carnitine works as an antioxidant to prevent the formation of ROS by removing hydrogen peroxide. L-Carnitine also increases the activity and level of antioxidant enzymes such as SOD and GSH-Px (Koberska & Yermishev, 2017).

The enzyme superoxide dismutase (SOD) is an endogenous antioxidant that works by regulating ROS levels (Simajuntak & Zukham, 2020). Superoxide dismutase (SOD) is responsible for converting oxygen-free radicals into H₂O₂. H₂O₂ is highly toxic and immediately disposed of (Kürüm et al., 2019). Reactive Superoxide is a superoxide radical or anion produced when oxygen loses one of its electrons, superoxide dismutase (SOD) is responsible for catalyzing the change from superoxide to elemental oxygen and hydrogen peroxide (Fitriani et al., 2022).

The addition of antioxidants, such as SOD, into equine sperm, bull sperm, and buffalo semen has demonstrated a protective effect against the detrimental impacts of ROS and improves sperm motility and membrane integrity during the storage of sperm fluid or in its unfrozen condition (Perumal, 2014). The advantageous role of SOD in the preservation of semen can be attributed to its potent antioxidant properties (Cocchia, 2011).



MATERIALS AND METHODS

Research location: This research was conducted at the Regional Artificial Insemination Center (RAIC) of South Sulawesi and evaluated in the In Vitro Embryo Production Laboratory, LPPM Building, Hasanuddin University, Makassar.

Materials: A total of 4 Bali bulls were used in this research with 9 years, 70% alcohol, pH paper, straw, tissue, vaseline, distilled water, androMed®, 0.25 ml Mini Straw (Minitube Germany), liquid nitrogen, eosin-nigrosin, acridine orange (AO), HCl, 96% ethanol, Hypoosmotic Swelling Test (HOST), Formasaline, 0.9% NaCl, carnoy solution, SOD (Sigma-Aldrich, Cat no: S5395) and L-carnitine (Sigma-Aldrich, Cat no: C0158).

Tools: The tools used in this research are a goblet, liquid nitrogen container, coolbox, cover glass, Petri dish, scissors, hand counter, refrigerator, filling and sealing machine, micropipette, Olympus CX33 trinocular microscope with Olympus EP50 camera, Zeiss AX10 Imager A2 fluorescence microscope with Zeiss AxioCam HRC camera, glass object, sperm tube, artificial vagina, and water bath.

Semen procedure and evaluation: Semen collection was used by the artificial vaginal method. Semen collection was then evaluated macroscopically, which included volume, consistency, color, and pH, as well as microscopic evaluation, which included mass movement, motility, and concentration. The eligible fresh semen will be further processed to the dilution stage by adding AndroMed® Diluent (Germany Minitube) which is one of the commercial semen diluents that does not contain egg yolk. This commercial diluent not only eliminates the risk of contamination from microorganisms associated with egg yolk but also offers convenience due to its availability in ready-to-use packages. The formulation of AndroMed® includes Tris hydroxy-aminomethane as a buffering agent, sugar serving as an energy source, glycerol functioning as a cryoprotectant, and antibiotics to inhibit bacterial growth (Juniandri et al., 2014). Furthermore, the addition of antioxidants was divided into two stages. The first stage involved four different concentrations of SOD (control, 0.5 mM, 1 mM, 1.5 mM) and four different concentrations of L-Carnitine (control, 0.5 mM, 1 mM, 1.5 mM) and the second stage involved evaluating three treatment groups, SOD 1.5 mM, L-Carnitine 1 mM, and a combination of both (SOD 1.5 mM and L-Carnitine 1 mM). Each treatment was repeated four times, then the diluted semen was filled into the straw (filling) as much as 0.25 ml/straw then continued with the process of sealing the straw (sealing) to prevent leakage during storage. The straws were equilibrated in a cooling chamber at 4-5 °C for a duration of three hours. Pre-freezing was performed by positioning the straws in nitrogen vapor within a Styrofoam container

filled with liquid nitrogen to a height of 7 cm, ensuring that the straws were suspended 4 cm above the liquid nitrogen surface for a period of ten minutes. Finally, the straws were frozen and stored in a container at a temperature of -196 °C, in accordance with the findings of Rangkuti et al. (2021).

Post-Thawing evaluation: Thawing uses warm water at 37°C for 30 seconds (Sukmawati et al., 2014). Post-thawing evaluation includes motility, abnormality, viability, plasma membrane integrity (MPU), Acrosome integrity (TAU), and DNA integrity (Gustina et al., 2024). Motility evaluation was performed following the procedure reported previously by (Priyanto et al., 2015). Motility was assessed subjectively by looking at the number of spermatozoa that moved straight ahead (progressive) with an assessment of 0-100% on a 5% scale. Viability evaluation was carried out using eosin-nigrosin dye by making screw preparations which were air-dried for 15-20 seconds. The preparations were observed under a light microscope using 200 times magnification. The live sperms (transparent) and dead (purple) spermatozoa were counted in 10 fields of view (Priyanto et al., 2015). The abnormality of spermatozoa was evaluated using eosin-nigrosin dye. Observations were made on a minimum of 200 spermatozoa by looking at the head, neck, and tail of the spermatozoa. There are five categories of abnormal spermatozoa, namely, no tail, abnormal head, abnormal tail shape with cytoplasmic droplet at the proximal part, and abnormal tail shape at the distal part of the droplet (Susilawati, 2011). Plasma membrane integrity was evaluated using the Hypoosmotic Swelling Test (HOSTest) method. The evaluation was performed under an Olympus CX33 microscope with an EP50 camera at 200 times magnification. Spermatozoa with plasma membrane integrity was characterized by a coiled or bulging tail, while plasma membranes without integrity were characterized by a straight tail. MPU evaluation is performed on 5 times the field of view or at least 200 spermatozoa cells (Priyanto et al., 2015). Evaluation of acrosome integrity was performed using a formalin solution (1% formalin; 0.9% physiological NaCl) following the procedure reported previously by Cahya et al. (2017). DNA integrity was evaluated with a Zeiss Axio Imager A2 fluorescence microscope with a Zeiss AxioCam HRC camera using acridine orange (AO) dye following the procedure reported by Adiputra et al. (2022). DNA integrity will emit fluorescent orange, while unfragmented spermatozoa will produce green (Handayani et al., 2021).

Data analysis: Data obtained is processed using Ms. Office Excel and analyzed in the IBM SPSS Statistical program for Windows, version 22 (IBM Corp., NY, USA). A post hoc Tukey test was conducted to determine the significant differences among the four treatments, both with and without the addition of antioxidants. Differences were



considered statistically significant at a P-value of less than 0.05.

Research design: This research is an experimental study using a completely randomized design (CRD) conducted in two stages. The first stage consisted of introducing four distinct concentrations of superoxide dismutase (SOD) (control, 0.5 mM, 1 mM, 1.5 mM) alongside four varying concentrations of L-Carnitine (control, 0.5 mM, 1 mM, 1.5 mM). The second stage involved the evaluation of three treatment groups: SOD at 1.5 mM, L-Carnitine at 1 mM, and a combination of both SOD (1.5 mM) and L-Carnitine (1 mM). Each treatment was replicated four times.

RESULTS

Fresh semen quality: Fresh semen quality evaluation is a prerequisite to determine whether the collected semen is eligible for further processing into frozen semen. The quality of fresh semen of Bali bull in this research can be seen in Table 1.

Table 1. The quality of fresh semen Bali bull.

Parameters	Quality of Semen
Macroscopic	
Volume (Mean±SD)	7.37±1.10
Consistency	Tick Milky
Color	Creamy
pH (Mean±SD)	6.40±0.00
Microscopic	
Mass Movement	+++
Motility (%±SD)	70.00±0.00
Concentration (x10 ⁹)	1.281±0.267

Based on macroscopic evaluation, the volume, consistency, color, and pH of semen in this research were normal. The same thing in the microscopic evaluation showed that the mass movement, motility, and concentration of Bali cattle semen collected were in the normal category. The findings of the research indicated that the quality of fresh Bali bull semen was suitable for processing into frozen semen.

Addition of Antioxidants Superoxide Dismutase (SOD) and L. Carnitine: Evaluation of frozen semen quality is something that must be done to determine whether the

Table 2. The quality of the frozen semen with the addition of antioxidant Superoxide Dismutase (SOD).

Treatments	Quality of Semen					
	Motility (%)	Abnormality (%)	Viability (%)	Membrane Integrity (%)	Acrosomal Integrity (%)	DNA Integrity (%)
Control	34.38±6.23 ^a	15.20±8.99	58.32±5.03 ^a	71.72±3.95 ^a	62.75±4.88 ^a	7.13±3.79 ^a
SOD 0.5 mM	38.75±6.94 ^{ab}	14.20±4.48	62.72±5.39 ^{ab}	75.19±3.77 ^{ab}	67.80±6.43 ^{ab}	2.48±2.44 ^b
SOD 1 mM	42.5±4.63 ^{bc}	8.30±4.62	66.54±2.54 ^{bc}	78.67±3.69 ^{bc}	72.48±5.20 ^{bc}	2.44±1.86 ^b
SOD 1.5 mM	48.13±4.58 ^c	7.30±2.66	70.56±5.45 ^c	81.09±3.64 ^c	74.08±2.66 ^c	2.39±1.80 ^b

Note: Different superscripts in the same column show significant differences (P<0.05)

Table 3. The quality of frozen semen of Bali bull with the addition of antioxidant L. Carnitine.

Treatments	Quality of Semen					
	Motility (%)	Abnormality (%)	Viability (%)	Membrane Integrity (%)	Acrosomal Integrity (%)	DNA Integrity (%)
Control	32.00±5.70 ^a	13.44±3.62 ^a	37.12±10.18 ^a	72.58±5.51 ^a	62.32±3.40 ^a	1.45±1.35 ^a
L-Carnitine 0.5 mM	42.00±10.95 ^{ab}	10.19±3.24 ^{ab}	69.91±12.17 ^b	78.57±1.61 ^b	69.42±1.35 ^{ab}	4.35±3.57 ^b
L-Carnitine 1 mM	47.00±7.58 ^b	7.19±3.12 ^b	74.55±6.22 ^b	84.95±3.10 ^c	75.12±9.10 ^b	1.36±0.85 ^a
L-Carnitine 1.5 mM	44.00±4.18 ^{ab}	9.06±4.03 ^{ab}	73.59±4.12 ^b	82.43±5.22 ^{bc}	74.21±5.01 ^b	2.48±1.48 ^{ab}

Notes: Different superscripts in the same column show significant differences (P<0.05)

Table 4. The quality of frozen semen Bali bull with the addition of antioxidants Superoxide Dismutase (SOD) and L-Carnitine.

Treatments	Quality of Semen					
	Motility (%)	Abnormality (%)	Viability (%)	Membrane Integrity (%)	Acrosomal Integrity (%)	DNA Integrity (%)
SOD 1,5 mM	48.75±8.54	8.75±3.23	73.13±9.44	79.17±6.29	78.75±3.23	1.95±1.65
L-Carnitine 1 mM	47.50±6.45	7.00±1.68	84.63±6.17	88.67±1.26	79.13±2.84	1.20±1.03
SOD 1.5 mM + L-Carnitine 1 mM	48.75±2.50	8.13±1.97	84.45±7.68	89.33±3.88	80.63±6.57	1.13±0.85



frozen semen produced is eligible and suitable for use in artificial insemination programs. The quality of frozen semen obtained in this research with the addition of antioxidant superoxide dismutase (SOD) can be seen in Table 2.

The addition of 1.5 mM SOD significantly enhanced motility, viability, plasma membrane integrity, and acrosome integrity ($P < 0.05$) when compared to the control, as well as to 0.5 mM SOD and 1 mM SOD. The same thing in the addition of SOD 1 mM was significantly higher ($P < 0.05$) than the control, but the addition of SOD 1 mM was not different from the addition of SOD 0.5 mM and the addition of SOD 0.5 mM was also not different from the control. While the abnormality was not different in each treatment, however, there was a noticeable trend indicating a reduction in abnormalities with increasing concentrations of SOD, (from 0.5 mM to 1.5 mM).

Based on the results of the research, the semen quality of Bali bull after the addition of L-Carnitine is presented in Table 3.

The addition of 1 mM of the antioxidant L-Carnitine significantly enhanced motility, viability, plasma membrane integrity, and acrosome integrity ($P < 0.05$) when compared to the control group. However, the control treatment did not show a significant difference ($P > 0.05$) when compared to the addition of 0.5 mM and 1.5 mM of L-Carnitine.

The quality of frozen semen Bali bull after the addition of antioxidants SOD and L-Carnitine and their combination is presented in Table 4.

The addition of 1.5 mM SOD, 1 mM L-Carnitine, and the combination of 1.5 mM SOD and 1 mM L-Carnitine did not affect semen quality, but the addition of the combination of 1.5 mM SOD and 1 mM L-Carnitine showed a trend towards higher MPU and TAU and lower DNA integrity.

DISCUSSION

The quality of fresh semen: The findings of the research (Table 1) indicated that the quality of fresh Bali bull semen was suitable for processing into frozen semen. [Feradis \(2010\)](#) reported that the volume of semen can be known by reading the scale found on the collection tube, the volume of bull semen per one ejaculation ranges from 5-8 ml. Furthermore, [Suteky et al. \(2017\)](#) explained that the volume of fresh semen of Bali cows in RAIC in Bengkulu ranged from 5-5.4 ml. The consistency or degree of viscosity of the semen can be checked by slowly vibrating the tube containing the semen. Fresh semen consistent in the medium thick range is included in the normal category ([Rangkuti et al., 2021](#)). [Bria et al. \(2022\)](#) The normal color of fresh Balinese cattle semen is beige, further [Lestari et al. \(2014\)](#) explained that the beige color of semen is caused by

the presence of riboflavin from the secretion of vesicular glands and the number of spermatozoa in semen, resulting in more concentrated colored semen. Semen quality affects the color of the semen, where normal semen is beige, while yellowish green semen means contaminated with *Pseudomonas aeruginosa* bacteria, and if the semen is red, it means it is contaminated with blood. [Mila et al. \(2021\)](#) state that the normal pH of cow semen ranges from 6.2 to 6.8 and is one of the parameters used to assess the quality of fresh semen. Furthermore, [Fitriani et al. \(2022\)](#) stated that the pH value of semen is influenced by seminal plasma fluid in semen which causes the semen to become more alkaline.

Microscopic evaluation of fresh semen in this research showed that the mass movement, motility, and concentration of Bali bull semen collected were in the normal category and could be further processed into frozen semen. [Rangkuti et al. \(2021\)](#) reported that mass movement ranging from (++) - (+++) shows the presence of large, dark, and active waves like clumps of black clouds that move quickly and move places. The motility of fresh semen suitable for processing into frozen semen has a minimum motility of 70% ([BSN, 2017](#)). [Ismaya \(2014\)](#) further explains that high sperm concentration (1,000-2,000 million sperm/ml), while dilute or cloudy indicates medium concentration (500-900 million sperm/ml), liquid or slightly cloudy indicates low concentration (100-400 million sperm/ml), and clear indicates low concentration (less than 100 million sperm/ml).

Addition of Antioxidants Superoxide Dismutase (SOD) and L. Carnitine: Evaluation of post-thawing spermatozoa motility is one of the parameters used to determine semen quality. Based on the results of this research (Table 2), shows that the addition of SOD 1.5 mM is able to increase motility due to the presence of antioxidants that protect sperm from free radicals such as Reactive oxygen species (ROS). This is quite similar to the viewpoint expressed by [Perumal \(2014\)](#), who stated that SOD at a concentration of 100 U/mL enhances sperm motility during preservation and exhibits antioxidant properties. This results in improved membrane fluidity and greater dehydration at lower temperatures, thereby allowing spermatozoa to survive throughout the preservation process. During the freezing process, there are significant changes in osmolarity and temperature that trigger the production of ROS which have an impact on spermatozoa motility ([Wijayanti et al., 2023](#)). Reactive oxygen species are produced excessively in the body, triggering oxidative stress in cells so that antioxidants such as SOD are needed to protect these cells ([Widayati, 2012](#)).

The viability of spermatozoa determines the quality of semen because the more live spermatozoa, the greater the chance of fertilization ([Tanii et al., 2022](#)). The percentage of spermatozoa viability is influenced by metabolic



processes and cellular activity of spermatozoa which are directly related to the plasma membrane (Ardhani et al., 2020). Damaged spermatozoa cell membranes will absorb color during viability testing (Gustina et al., 2020). In addition, nutrients in the diluent during the storage process also affect spermatozoa viability (Fernandes et al., 2022).

The plasma membrane is directly related to the metabolism and cellular activity of spermatozoa. The addition of 1.5 mM SOD in this research was able to increase the MPU of Bali bull spermatozoa allegedly due to the presence of antioxidants that protect cells from the attack of free radicals that damage the membrane. During the freezing process, extreme changes in temperature and osmolarity occur and trigger the production of ROS that damage the lipid composition of the plasma membrane (Wijayanti et al., 2023). Lipid peroxidation in the plasma membrane increases if the concentration of ROS produced is higher (Lestari et al., 2022). The increase in ROS causes an oxidative stress imbalance, which is a situation where the concentration of ROS is higher than the total antioxidants in the body, so enzyme activity is needed to neutralize ROS so that the amount is reduced (Aprila et al., 2018). SOD enzyme is the first defense to protect cells from ROS activity (Astuti, 2012).

The acrosome integrity in this research increased with the addition of 1.5 mM SOD, indicating that the SOD added in the diluent protects the acrosome integrity from free radical attack that can have an impact on the damage of the acrosome integrity. Acrosome integrity can be affected by treatment, feed, environment, and semen handling processes (Anwar et al., 2015). The increase in frozen semen storage time, damage during handling, and the occurrence of cold shock during the freezing process are also factors in decreasing the percentage of TAU (Ardhani et al., 2020).

The percentage of DNA integrity in this research was lower in the addition of 1.5% SOD because SOD which acts as an antioxidant protects cells from oxidative stress and lipid peroxidation during the freezing process (Gustina et al., 2024). Free radicals such as ROS cause lipid peroxidation and membrane degradation resulting in DNA damage (Priyanto et al., 2018). The antioxidant content in SOD is able to protect cells from free radical attacks such as reactive superoxide radicals (Fitriani et al., 2022). Perumal (2014) states that the addition of exogenous SOD significantly enhances the morphological percentage of DNA, sperm viability, and the integrity of the plasma membrane (swelling tail).

The concentration of antioxidants added to the extender must be carefully considered, as high doses of antioxidants can adversely affect spermatozoa due to alterations in the physiological conditions of semen. The viability of spermatozoa tends to improve with an increase in the dosage of antioxidants in the extender. However,

exceeding the necessary amount of antioxidants can be toxic to spermatozoa. Excessive superoxide dismutase (SOD) may lead to detrimental effects on the development or maturation of spermatozoa, as well as damage to sperm cells, ultimately resulting in a decreased fertilization potential of the sperm (Perumal 2014).

Based on the results of this research (Table 2), it shows that the addition of SOD does not affect the abnormality of Bali cattle spermatozoa. This indicates that the addition of SOD up to 1.5 mM is not able to reduce spermatozoa abnormality. However, spermatozoa abnormality in this study was <20% which indicates that the frozen semen produced can be used for artificial insemination (Ardhani et al., 2020). An increase in spermatozoa abnormality can occur when produced into frozen semen due to differences in osmosis during dilution, the occurrence of cold shock during the cooling process, and the preparation of a review that causes the breakage of the spermatozoa tail (Wijayanti et al., 2023).

L-Carnitine is a vitamin-like component synthesized from two essential amino acids, lysine, and methionine, which have antioxidant characteristics. L-Carnitine also functions indirectly in the maturation process of spermatozoa in sertoli cells by increasing energy. Based on the results shown (Table 3), the addition of L. Carnitine 1 mM increases the motility of frozen semen of Bali bull. This result is consistent with the study by Darussalam (2020), which indicated that the inclusion of 1 mM L-Carnitine led to a notable improvement in sperm motility compared to the control group. Additionally, semen treated with 1 mM L-Carnitine showed a reduced proportion of dead sperm in comparison to both the control and other concentrations of L-Carnitine. The increase in motility after the addition of L. Carnitine 1 mM is thought to be due to the influence of antioxidants that play a role in helping the metabolic process of spermatozoa. Balogun et al. (2022) reported the addition of L. Carnitine plays a role in the metabolic process of spermatozoa due to the role of antioxidants and the role of carnitine in producing ATP for sperm motility. L. Carnitine will facilitate the transportation of fatty acids to the mitochondria to carry out the β -oxidation mechanism so that ATP is formed as an energy source (Darussalam et al., 2020). Spermatozoa move using energy produced by mitochondria, namely chemical energy which is converted into kinetic energy through the enzyme ATP (Antari & Damayanti, 2020).

The findings of this research can be observed in Table 3. The results of this research showed that the addition of L. Carnitine 1 mM decreased the percentage of frozen semen abnormality in Bali bull. This indicates that the addition of L. Carnitine 1 mM can reduce the abnormality of frozen semen of Balinese cattle. Saputra et al. (2024) reported that the administration of L. Carnitine in excessive amounts can reduce the quality of spermatozoa, including damage to



sperm morphology. The addition of 1 mM L. Carnitine can increase sperm viability due to the influence of antioxidants in L. Carnitine. [Solarska et al. \(2010\)](#) found that incorporating L-Carnitine (1 mM) into extenders for Pasundan bull semen significantly improves sperm viability by $43.95 \pm 0.42\%$, achieving a total of 108. As an antioxidant, L-Carnitine plays a crucial role in safeguarding the plasma membrane of spermatozoa, primarily owing to its elevated concentrations of unsaturated fatty acids ([Aitken et al., 2010](#)). Antioxidants in L. Carnitine prevent the formation of free radicals in sperm production ([Antari & Damayanti, 2020](#)). Furthermore, [Darussalam et al. \(2020\)](#), reported that spermatozoa viability is influenced by the integrity of the plasma membrane. One that causes damage to the plasma membrane is the presence of ROS. L-Carnitine is known to bind ROS so that it can maintain membrane integrity. In addition to death due to cold shock, thawing is also one of the causes of spermatozoa death due to the amount of lactic acid from spermatozoa metabolism that cannot be oxidized. The accumulation of lactic acid results in an increase in the acidity of the solution which is bad for spermatozoa because it is toxic. The fulfillment of antioxidant needs can maintain the percentage of spermatozoa during the freezing process, but the provision of excessive amounts of L. Carnitine is toxic, so it can reduce the quality of spermatozoa ([Saputra et al., 2024](#)). The plasma membrane integrity of frozen semen Bali bull increased with the addition of L. Carnitine 1 mM, presumably due to the activity that protects cells from free radical damage. Plasma membranes containing unsaturated fatty acids and phospholipids are very vulnerable to the presence of ROS that can break fatty acid chains into toxic compounds ([Antari & Damayanti, 2020](#)). Antioxidants in L-Carnitine will prevent the formation of free radicals that result in the formation of lipid peroxides resulting in damage, but excessive levels of antioxidants will cause oxidation of sperm membranes ([Saputra et al., 2024](#)). [Lisboa et al. \(2022\)](#) explained that the addition of L-Carnitine and acetyl-L-Carnitine, alone or in combination increased spermatozoa motility and plasma membrane integrity of horse sperm after refrigerated storage at 5 °C for up to 48 hours. The acrosome integrity has a very important role in the fertilization process because it protects the acrosome of spermatozoa cells containing enzymes so that they can penetrate the zona pellucida ([Antari & Damayanti, 2020](#)). The acrosome integrity of frozen semen of Bali bull increased with the addition of 1 mM L. Carnitine. This suggests that L. Carnitine 1 mM can protect spermatozoa acrosomes through antioxidant activity. [Ardhani et al. \(2020\)](#) explained that the freezing process can cause damage to the acrosome due to ice crystals that form when cell dehydration occurs. Furthermore, [Alhuur et al. \(2020\)](#) reported that during the

freezing process, cells will experience cold shock so that enzymes will leave the cell body which can result in failure of the fertilization process.

The percentage of DNA integrity in this research decreased with the addition of 1 mM Carnitine. This is due to antioxidant activity that can prevent the formation of free radicals that result in the formation of lipid peroxides ([Saputra et al., 2024](#)). [Akmal \(2016\)](#) explains that DNA integrity occurs due to damage to the DNA strands. Damage to spermatozoa DNA will have an impact on reducing spermatozoa fertility and affect the process of fertilization and embryo development ([Priyanto et al., 2018](#)). Damage to spermatozoa DNA is the cause of pregnancy failure in livestock around 6.9% ([Meseguer et al., 2011](#)). In the DNA oxidation process, damaged spermatozoa DNA will cause errors in the DNA replication process resulting in mutations ([Akmal, 2016](#)).

The enzyme superoxide dismutase (SOD) is an endogenous antioxidant that works by regulating ROS levels ([Simanjuntak & Zulham, 2020](#)) while carnitine is an amino acid derivative that functions to increase energy production and act as an antioxidant for sperm cells. Superoxide Dismutase is responsible for catalyzing the change from superoxide to oxygen and hydrogen peroxide elements ([Fitriani et al., 2022](#)) while L-Carnitine has an important role in sperm cell energy metabolism. Sperm cells require energy generated from mitochondria for spermatozoa motility ([Balogun et al., 2022](#)).

L-Carnitine is found in high concentrations in the epididymis, the organ where sperm cells mature, increases motility, and utilizes fatty acids and phospholipids as energy substrates, suggesting that L-Carnitine has an important role in sperm cell energy metabolism. Sperm cells require energy generated from mitochondria for spermatozoa motility ([Balogun et al., 2022](#)). The way L-Carnitine works as an antioxidant is to prevent the formation of ROS by removing hydrogen peroxide. L-Carnitine also increases the activity and level of antioxidant enzymes such as SOD and GSH-Px ([Koberska & Yermishev, 2017](#)). The enzyme superoxide dismutase (SOD) is an endogenous antioxidant that works by regulating ROS levels ([Simanjuntak & Zulham, 2020](#)). Superoxide dismutase (SOD) is responsible for converting oxygen free radicals into H_2O_2 , which is highly toxic and immediately disposed of ([Kürüm et al., 2019](#)). Reactive Superoxide is a superoxide radical or anion produced when oxygen loses one of its electrons, Superoxide Dismutase / SOD is responsible for catalyzing the change from superoxide to elemental oxygen and hydrogen peroxide ([Priyanto et al., 2018](#)).

Conclusion: The research findings suggest that the addition of 1.5 mM SOD and 1 mM L-Carnitine can improve the quality of frozen semen in Bali bull, indicating



its possible utility in enhancing artificial insemination programs.

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SDGs addressed: Zero Hunger, Responsible Consumption and Production, Life on Land .

Policy referred: Conservation and sustainable use of indigenous cattle genetic resources; Improvement of reproductive efficiency and productivity through AI technology using high-quality frozen semen; Compliance with national quality standards (SNI 4869-1:2017); Promotion of research and innovation in semen processing and antioxidant use to enhance AI success rates and livestock productivity.

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